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PRINCIPAL INVESTIGATOR: David N. Krag, M.D.

CONTRACTING ORGANIZATION: University of Vermont
Burlington, Vermont 05405-0160

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13. ABSTRACT

Our lab is attempting to identify small ligands which bind specifically to breast tumor cells for ultimate use in tumor-targeted therapy. Our major target is the extracellular domain (ECD) of the ErbB2 growth factor receptor, which is overexpressed on the tumor cells of many breast cancer patients. This year we have succeeded in purifying ErbB2-ECD in the form of an ECD-alkaline phosphatase fusion protein to a high degree of purity and in amounts adequate to easily perform ErbB2-ECD affinity purifications and binding assays for putative ligands. This year we used a phage-displayed peptide library we constructed previously as a source of small ligands to ErbB2-ECD. We performed extensive screening experiments with our library against ErbB2-ECD target protein presented in different forms, bound to various matrices, and using several types of elution mechanisms. Several consensus sequences were obtained, which suggests that we have obtained at least one ErbB2-ECD binder, as some of the consensus sequences obtained are from screens with only ErbB2-ECD in common in the target system. However, none of the clones were of high enough affinity to give a positive ErbB2-ECD ELISA result. Inspired by the recent success at Affymax [5,6], where small peptide ligands were identified to receptors similar to ErbB2, we have begun construction of several new libraries similar to those of Affymax. These new libraries will be more likely to allow us to identify high affinity small ligands to potential tumor targets such as ErbB2.

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PAUL A. LUCY MD 9/29/97

PI - Signature Date

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Introduction

The major problem in the treatment of breast cancer is the lack of a therapy which specifically attacks tumor cells. Our lab is attempting to identify small ligands which bind specifically to tumor cells for ultimate use in tumor-targeted therapy. Although many tumor-specific antibodies have been identified, coupled to cytotoxic agents or alone, the performance of Abs in clinical trials has been somewhat disappointing. Ligands much smaller than antibodies (1-2 kD) will likely have pharmacokinetics and tumor penetration superior to that of Abs or Ab fragments, are much less likely to be immunogenic, and may therefore allow the development of more effective targeted therapeutics. One example of the use of small ligands (8 and 12 mer peptides) in targeting tumors has been reported by Renschler et al. [1,2,3] who used phage displayed small peptide libraries to identify peptides which bind to the antigen binding receptor of B-lymphoma cells and induce apoptosis *in vitro*.

Large libraries of small compounds are a rich source of potential small ligands to tumor targets and other macromolecules. Within the last seven years, several types of these libraries have been constructed and used successfully to isolate ligands to selected targets. Libraries can consist of millions or even billions of different peptides, oligonucleotides (aptamers), or synthetic chemical compounds. The construction of libraries like these, and their use in the identification of specific ligands, also called combinatorial technology, has revolutionized the field of drug discovery [4].

We have constructed a phage-displayed random peptide library containing over 20 million different peptides which have the potential of being constrained by a disulfide loop by flanking the random peptides with cysteine codons. Libraries containing peptides flanked by cysteines, with the potential of forming a disulfide loop, often yield more binders to targets than linear libraries [5,6]. Although such constrained libraries will not represent as many different spatial conformations as a linear library, any binder identified will more likely have higher affinity than a linear binder, as linear peptides can usually assume many conformations other than the active binding conformation. Constraints introduced by the presence of a beta-turn inducing Gly-Pro sequence have also been used successfully [5,6].

Small peptide ligands which we identify to tumor targets will most likely need to be modified and/or used as a prototype in order to develop a small molecule which will be effective *in vivo*. We have already identified a small peptide ligand to one tumor target, Grb2, and our collaborators have modified it chemically such that it retains binding activity in cell lysates [7]. However, as described in two exciting reports in *Science*[5,6], Affymax (Palo Alto, CA) has identified peptides to two different cell receptors which have significant half-lives and activities *in vivo*.

The primary tumor target for which we are seeking a small ligand, is the class I tyrosine kinase growth factor receptor ErbB2. ErbB2 is a promising target as it is overexpressed on the tumor cells of approximately 30% of breast cancer patients, appears to be homogeneously expressed by all cells within a tumor, and is found only minimally on normal cells. A small ligand to ErbB2 could be used to specifically delivery cytotoxic compounds to tumor cells. An especially intriguing potential use of ErbB2 is for the specific delivery of therapeutic antisense oligos [8,9].

During the first two years of this project we attempted to identify small ligands to ErbB2 from our random peptide library using ErbB2 presented by ErbB2-overexpressing cells or ErbB2 from

cell extracts bound to a matrix via an Ab to the intracellular domain. We identified several consensus peptides this way, although we were not able to demonstrate whether or not these peptides bound specifically to ErbB2. A major limitation in our progress so far has been that we have not had an adequate source of pure ErbB2 in order to easily perform our panning and assay experiments. However, we have recently succeeded in purifying sufficient quantities of very pure ErbB2 ECD, as discussed in the body of this report, which has greatly facilitated our progress.

In addition, the two recent reports from Affymax have been extremely inspirational for our own work as they describe the identification of small peptide ligands to the extracellular domains of two type I cell surface receptors which have a general structure very similar to ErbB2: an N-terminal extracellular domain (ECD), a single helical transmembrane domain, and a C-terminal intracellular domain (ICD). Binding of the natural ligands to these two receptors, (erythropoietin receptor and thrombopoietin receptor), induces activation via dimerization. A similar mechanism has been proposed for ErbB2 activation. The peptide ligands they identified (and modified slightly) were unrelated to the much larger natural ligands, had high affinity (nanomolar to picomolar), and had significant agonist activity *in vivo* to these therapeutically important targets. This elegant work is especially exciting for our lab in that the whole premise of our project is no longer a "hypothesis": small, specific peptide ligands to targets which are only known to bind much larger ligands can not only be isolated, but these small peptide ligands can bind with high affinity and maintain activity *in vivo*. While combinatorial technology was quite new when the initial grant proposal for this project was submitted, the technology has grown exponentially in just a few years. The work of other groups, particularly that of Affymax, who has been kind enough to supply us with many valuable vectors, bacterial strains, and detailed protocols, has expanded the initial technology greatly, and has allowed us to incorporate many new ideas and techniques into our own work, which, along with our recent purification of ErbB2, should allow us to more rapidly achieve our goals. Some of the highlights of these new ideas, schemes and techniques are listed below:

- Two types of peptide libraries other than phage display have emerged which have both advantages and some disadvantages over phage display:
    - Peptides-on-plasmids [10]: This system allows the display of library peptides from a Lac repressor protein, which binds to a plasmid engineered to code for the same Lac repressor protein (four copies of peptide displayed per plasmid), thus physically linking the displayed peptides with their coding DNA. This system should not be restricted by the biological biases known to occur in phage display [11]. In addition, because the plasmids are much smaller than phage particles, many more different peptides can be screened at one time. And importantly, peptides are displayed from the C-terminus of the protein, while phage displayed peptides are displayed from the N-terminus of phage coat proteins. This is of obvious importance if the most active portion of potential peptide ligands is located at either their C-terminus or N-terminus. The system is somewhat more difficult technically than phage display.
    - Polysomes [12,13]- This system allows the display of library peptides directly from the mRNA which codes for them. Amplification of binding peptides occurs via synthesis of the corresponding cDNA and PCR. The principle advantage of this system is that the

complexity of the library can be huge- up to 10^{13} to 10^{14}. The technique is not as straight-forward as phage display.

- A "portfolio" of different types of libraries [14] is important, as ligands are found to some targets with only one type of library. Besides the obvious differences in phage displayed libraries vs. peptides-on-plasmids libraries, or even gene III vs. gene VIII phage display system, there are differences even between gene III systems. For example, Cwirla/Dower's gene III system [15] displays peptides at the extreme end of the N-terminus of the gene III protein, where Smith's gene III system [16] contains a few amino acids N-terminal to the random peptide sequence. Because of biological biases, one of either of these gene III systems may work better for certain peptide ligands.

- In the case of combinatorial technology, bigger is definitely better. When phage displayed peptide libraries were first reported, libraries were often the size of ours, on the order of 10^7. Peptides libraries as large as 10^{11} are now being reported [17]. Such huge libraries allow a sampling of a much greater percent of the all the possible number of spatial conformations of longer peptide libraries.

- It is often better to begin screening with a library which presents multiple copies of the potential ligand on each DNA package (for example, the gene VIII phage display system or the peptides-on-plasmids system). Because of an avidity effect, a ligand is more likely to be discovered, albeit with perhaps lower affinity than ligands isolated from a monomeric presentaion system. The consensus sequences identified from these initial screens are then used to design mutagenesis libraries presented on a (relatively) monomeric system such as the gene III phage display system or the headpiece dimer version of the peptides-on-plasmids system [18]. Screenings of these mutagenesis libraries are more likely to yield much higher affinity binders.

- Random libraries are not only usually more successful when disulfide loops are incorporated into the design- libraries designed with random "flanker" regions of 3 or 4 amino acids outside the disulfide loop have yielded binders when disulfide loops alone failed [5].

- Building block variety may be optimal. For example, G-protein coupled receptors are now known to bind ligands that must be amidated at the C-terminus. C-amidated peptide libraries are more likely to yield small ligands to these receptors. Synthetic and polysome peptide libraries can also be constructed with unnatural amino acids. Hits from these libraries may have *in vivo* activity without further modification.

- Several techniques used by Affymax were necessary to obtain any hit or to increase the affinity of binders isolated:

    - Presentation of the receptor ECD was via an Ab bound to a matix. Such a presentation may simulate dimerization spatially and may be required to obtain ligands.

    - In the case of the initial EPOR screens, the EPOR had to be engineered to contain a thrombin cleavage site. Only elutions by thrombin cleavage were successful in yielding binders.

    - For the high affinity screens, the target was preeluted with natural ligand in order to compete off lower affinity binders so that only high affinity sequences would be identified.

Many of these new techniques will be incorporated into our own work in the near future. We have already begun construction of several new libraries incorporating these new library designs, as described in the conclusions section of this report.

As mentioned in previous reports, from our original peptide library we have also identified small ligands to streptavidin, a monoclonal Ab to ErbB2, and the SH2 domain of Grb2 and have confirmed their binding specificity by ELISA. The ligands to Grb2 were identified as part of a collaboration with C. Richter King at Georgetown who is attempting to develop non-phosphorylated small ligand inhibitors to Grb2 , which is necessary for ErbB2 signal transduction. Grb2 inhibitors may therefore be useful for breast cancer therapy. We obtained a specific Grb2 SH2 domain binder in less than two weeks from our library. Investigators at the NCI, NIH have already developed the peptide into a stable peptidomimetic which blocks the function of Grb2 in cell lysates [7].

We are also collaborating with John Burke's "ribozyme" lab, and will use aptamer libraries to try to identify additional novel ligands to ErbB2. The aptamer library has already been synthesized and now that we have an adequate source of purified ErbB2, we are proceeding with ErbB2 screenings of the aptamer library.

Body

The first part of this year we focused our efforts on continuing the development of an efficient purification procedure for our primary target- the ECD of ErbB2. We tried several different methods, and finally elucidated a satisfactory protocol. We have used partially purified ErbB2 ECD as well as the highly purified ErbB2 ECD, presented in a variety of contexts, in exhaustive screening efforts to try to identify peptide ligands to ErbB2. We have identified several consensus sequences, some of them strong: for example, all the clones from one pan had an identical sequence. We have also used the newly available purified ErbB2 ECD to develop what seems to be a reliable ELISA. Unfortunately, despite extensive screening, none of the phage clones we isolated from ErbB2 ECD pans were positive in the ELISA assay. Although it is possible that the ELISA needs further improvement (discussed below), it is likely that none of our peptide-phage bind very tightly. Still, with strong evidence for several different consensus sequences- at least one of these consensus sequences identified is likely to bind to ErbB2, albeit weakly, for reasons discussed below. This consensus will be valuable in the isolation of higher affinity ligands, using mutagenesis libraries, described in detail below.

An interesting result of our purification efforts is the development of an assay for ErbB2 ECD dimerization.

We have also used our library to pan two other tumor targets: CEA and, in a collaboration with O.J. Finn at the University of Pittsburgh, the extremely promising breast cancer target, MUC-1 [19].

In addition, the Grb2-binding peptide we identified last year has been further developed into a peptidomimetic which blocks Grb2 function in cell extracts [7].

Purification of ErbB2 ECD-AP

As discussed in the last report, Matthias Kraus at the European Institute of Oncology, a leading investigator on ErbB receptors, has been kind enough to provide us with an genetically engineered mouse fibroblast line which secretes an ErbB2 ECD-alkaline phosphatase (AP) fusion protein into the media. Affinity purification with anti-AP Sepharose resulted in a many-fold purification of the ECD-AP, but left 2 or 3 contaminants in the preparations (Figure 1). Elimination of these few remaining contaminants proved to be quite difficult. Because the contaminants bound non-specifically to Sepharose, we had hoped that a post-incubation of the eluted ECD-AP prep with plain Sepharose would succeed in eliminating the contaminants. While the amount of contaminants did decrease with such an incubation, the amount of ECD-AP decreased as well. We also recloned the cell line in hopes of obtaining an increased yield of ECD-AP, and perhaps, a greater ratio of ECD-AP to contaminants. While some clones did appear to secrete more AP into the media, ECD-AP affinity preparations from these clones looked no different than the original preparations (Figure 1) and contained the same two major contaminants.

We proceeded to try the following protein purification techniques to completely purify the ECD-AP:

Preparative electrophoresis with a Biorad mini-prep cell. We loaded partially purified ECD-AP preps (eluates from the anti-AP Sepharose) onto both regular 7.5% SDS-PAGE tube gels, and native PAGE 5% tube gels. While separation on the SDS system was reasonable (Figure 2), there were still some lower MW contaminants (present in all fractions using the sensitive silver stain, and therefore probably a minor contaminant in the buffer or gel system) and the yield was not impressive. In addition, the recovered protein was likely to be denatured in this SDS system and would require renaturation steps which may not be successful. It might also require a lot of time to empirically determine an efficient renaturation protocol. The native gel system, besides also having lower MW contaminants, contained ECD-AP throughout many fractions (Figure 3), and thus did not effect efficient separation of ECD-AP from its contaminants.

Gel filtration and ion exchange FPLC: Again, using partially purified ECD-AP as a starting material, we loaded ECD-AP (1-5 micrograms) onto both hydrophobic interaction (butyl Sepharose, HiTrap, 1 ml, Pharmacia) and ion exchange (MonoQ FPLC, 5/5, Pharmacia) columns. The hydrophobic interaction column was completely unsuccessful as no ECD-AP was detected in any of the fractions. The ion exchange column, run with a 50mN Tris, pH 8.6 to 50 mM Tris, 1M NaCl, pH 8.6 gradient, was much more effective (Figure 4). Importantly, a quite pure band of ECD-AP was now detectable not only by silver stain, but by Coomassie as well. We later developed a more simple and efficient purification method. However, this method does work well and can be scaled up in the future if necessary.

Lectin-Sepharose affinity purification: Because the ECD of ErbB2 is known to be heavily glycosylated, at least in humans, we attempted to exploit this glycosylation (probably occurring in our mouse cells as well, although perhaps qualitatively different) for purification by incubating either "crude" ECD-AP sup or partially purified ECD-AP with Sepharose conjugated to three different lectins: ConA, wheat germ and lentil,. Each lectin bound many proteins in both the ECD-AP sup and the control 3T3 sup, with no difference noted between them, i.e. no ECD-AP band was noted (Figure 5). Because the lectins bound so many proteins, with no obvious

preference for ECD-AP, incubation of the crude sup with lectins did not seem to offer any advantage over our anti-AP Sepharose partial purification as a first step. We then incubated the partially purified ECD-AP preps with the three types of lectin-Sepharose as well as Protein A Sepharose (in case the major contaminants were of IgG origin) to see if the lectins or Protein A bound either ECD-AP or the contaminants preferentially. This was not the case (Figure 6). Not only were the preps not further purified, a lot of ECD-AP appears to have been lost along the way. For some reason, the ECD-AP eluted from Con A contained a plethora of bands. Either degradation had occurred, (although why degradation should only occur with ConA is not clear), or the ConA Sepharose contained many contaminants which were eluted off by the sugar solutions.

Electroelution from Native polyacrylamide gels: Different permutations of the Ornstein native PAGE system [20] were attempted until adequate separation of ECD-AP from the contaminants was achieved. We ended up being very lucky in this regard for two reasons: a 5% native gel allowed partially purified ECD-AP to enter and to run to the approximate center of the gel while the contaminants either did not enter at all, or ran off. Thus, complete purification was achieved just by running the native 5% gels. Secondly, apparent from both silver (Figure 7) and Coomassie stained (Figure 8) gels, and especially from Westerns (Figure 9), the native gel demonstrated the existence of ECD-AP in, apparently, both monomer and dimer form. One gel loaded with a lot of material suggested the presence of even higher multimeric forms (Figure 10). This is interesting in that others have speculated that the transmembrane domain of ErbB2 is involved in ErbB2 dimerization. Our fusion protein does not contain any TMD residues, which suggests that the ECD alone is sufficient for dimerization. Since AP can also form multimers, we ran pure AP on the gels, to see if both monomer and dimer forms were present (Figure 11). While we only saw one band, one can not rule out that this could be a dimer form, and that the monomer form had run off the gel. Indeed, a heavily staining dye front was consistent with this possibility. However, we reasoned that if the AP portion of the ECD-AP dimer was responsible for the dimers we saw on native gels, that preincubation of the ECD-AP preps with excess pure AP should cause at least partial dissociation of the dimers. Incubation of the ECD-AP dimers with an excess of AP for 40 min at RT had no effect on the amount of ECD-AP dimer present (Figure 12). In addition, no new band appeared as might be expected if an ECD-AP/AP complex had formed.

An interesting and relevant question regarding these results is: why are the dimers forming? It is supposed that ligand is necessary to stabilize the dimer complex. Could the 3T3 cells be producing a ligand to ErbB2 ECD? This would be surprising as the mouse cells are a fibroblast line and the receptor is normally found only on epithelial cells. The ligand might also be present in the calf serum present in the media. Also, importantly, if the dimers already have ligand bound, then they will not serve as effective "targets" as the site most likely to bind a peptide in our library will be masked. This could account for our difficulties in obtaining a binder. If this is the case, it will be important for us to remove the ligand before assembling our target presentation system. Data to support this possibility is discussed in the next paragraph.

Using a copper stain (BioRad) to visualize the ECD-AP bands on a 5% native gel (copper stain is not supposed to work on non-SDS gels, however, we were able to localize the edges of each band this way), we cut strips of gel containing both dimers and monomers of ECD-AP and electroeluted the protein (Figure 13). While both methods worked well, as we expected,

in removing the contaminants, the yield was only about 10%. Also, of great interest, relevant to the discussion above, was that the dimer, *but not the monomer*, upon re-running on a regular SDS gel, showed the presence of 2 lower MW bands. The lower of these bands (50 kD) is clearly different than the lower MW contaminant seen in partially purified preparations. We wonder if this lower band might represent a ligand which was bound by the native dimer form. However, it is not clear why these new "ligand" bands are not visible on SDS-PAGE of ECD-AP we elute directly from anti-AP Sepharose. The bands may represent degradation- however, it is not clear why only dimer would undergo slight degradation. These SDS gels of the ECD-AP bands eluted from native gels also serve to confirm that the two bands seen on the native gels are multimers of each other, as they are exactly the same MW on the SDS gels.

We believe that Westerns of ECD-AP run on native gels (Figure 9) will serve as an effective assay for ErbB2 dimerization. It will be of interest to see if any of our candidate peptide binders can inhibit or reverse dimerization. Of particular interest is the peptide we identified which had some homology with ErbB2 ECD, as discussed in last year's report. Other peptides, such as the RL2 peptide (described in our intial grant proposal as part of a collaboration with Marc Lippman at Georgetown), which represents a portion of the ECD, is also worth testing. Dr. Lippman has a panel of such peptides derived from the ECD and it seems worth testing them all for inhibition of dimeriztion in this simple assay. Since dimerization is presumed to be necessary for receptor activation, and the expression of ErbB2 is implicated with the pathogenesis of many breast cancers, such an inhibitor might serve as an effective therapeutic.

Affinity purification with anti-AP Sepharose from serum-free media sups. We determined that the two main contaminants originated from the serum in the media (Figure 14) and therefore attempted to establish our cell line in serum-free media (SFM). We initially attempted to grow our cells in SFM by "weaning" them off serum. This was unsuccessful- the cells did not grow well below 2.5% serum and ECD-AP prepared from this media was not improved in purity. We then prepared a SFM [21] which the ECD-AP secreting cells survived fairly well in, once they had been grown to sub-confluence in regular serum media. Affinity purifications with anti-AP Sepharose using SFM ECD-AP sup resulted in completely pure ECD-AP (Figure 15). Furthermore, scale up of cell growth to roller bottles resulted in a large increase in the amount of ECD-AP recovered (100ug/100ml) (Figure 16) from regular media with serum. We now routinely grow roller bottles to confluence, harvest that sup as a "regular" harvest (100 ug ECD-AP per 100 ml media), rinse the cells three times with saline, overlay the cells with SFM, and harvest when the media starts to become more acidic. We can overlay these cells for up to 3 or 4 harvests before they slough off. We only recover about a microgram for every 100 ml of SFM sup this way, but we only need target this pure for pans. 5 or 10 micrograms is usually sufficient for this purpose. More target is needed for ELISA assays, but the partially purified ECD-AP "regular" harvests, using the appropriate 3T3 control for duplicate samples, should be adequate for ELISA assays. We also plan to run ECD-AP purified from SFM on native gels to see whether there are dimer forms as in serum supplanted media.

Purification by "filtering" through YM100 membranes. We noticed during the course of attempting to concentrate our regular serum ECD-AP harvests with a YM100 Amicon system, that the filtrate not only, surprisingly, contained ECD-AP (MW 150kd), but that an affinity

preparation of ECD-AP from the filtrate, even more surprisingly, was now completely purified from the lower MW contaminants (Figure 17). (The retentate also contained ECD-AP, as expected). While the yield was low, it was comparable to SFM yields. The method is very simple, far less expensive than SFM, and is worth further exploration.

ErbB2 ECD-AP Pans

We have used the ErbB2 ECD-AP purified as described above to perform 9 different ErbB2 pans this year with our peptide library. The ECD-AP was presented on several different matrices and in several different presentation contexts as follows:

1) 2-5-97 Pan: ECD-AP was presented via a noncovalent linkage to anti-AP antibody which was covalently bound to Sepharose. Approximately 10 ug of ECD-AP attached to 5 ul of anti-AP Sepharose was used per pan. The phage (about $1x10^{11}$) were "precleared" by incubation with anti-AP Sepharose before adding the unbound phage to ECD-AP/anti-AP Sepharose. Phage were eluted successively with both a low pH (2.3) buffer and a high (pH 12) buffer.

2) 3-16-97 Pan: ECD-AP was presented on nitrocellulose (NC) paper by cutting out the appropriate sections of a native ECD-AP Western blot. Both monomer and dimers sections were used. Elutions were performed with both a high pH buffer (first), followed by a low pH elution.

3) 4-1-97 Pan: Partially purified ECD-AP was plated directly onto polystyrene wells in 96 well NUNC Maxisorb plates as was control protein isolated from elution of anti-AP Sepharose incubated with 3T3 sups only. Phage were precleared on the control protein wells before incubation with ECD-AP. These control wells were also eluted to moniter for enrichment. Elution was with both low and high pH buffers.

4) 4-16-97 Pan: ECD-AP was presented on NC as on 3-16-97 except this time phage were incubated separately with the dimer and monomer form of ECD-AP. Elution was with both high and low pH buffers. After the first pan, half the amplified phage were incubated with a control, blank piece of NC to moniter for enrichment.

5) 5-12-97 Pan: Highly purified ECD-AP was covalently coupled to Sepharose using NHS coupling chemistry. Elution was with high pH buffer only. During the third and last pan, half of the input phage were incubated with an anti-AP Sepharose control to moniter for enrichment.

6) 5-26-97 Pan: Highly purified ECD-AP and control AP was covalently coupled to tosylactivated Dynabeads M-280 (Dynal) as per the manufacturer's instructions. A magnet was used to separate the beads from incubation mixtures and wash fluids. Input phage were precleared with the AP-Dynabeads and, for the last pan only, the AP preclear beads were eluted to moniter for enrichment. Elution was with high pH buffer only.

7) 7-21-97 Pan: Similar to the 4-1-97 pan except highly purified ECD-AP was plated on polystyrene wells in 24-well plates to allow for incubation of a less viscous phage solution which may allow more rapid binding. Free AP was added to the phage solution to eliminate binding of AP-binding phage in the library to the AP portion of the ECD-AP bound to the

polystyrene. Elution was performed first with a cocktail of two different monoclonal antibodies which see two different epitopes on the ErbB2 ECD, followed by a high and low (in that order) pH elution. For the last pan, half the input phage were incubated with and eluted from a control (AP only) well to moniter for enrichment.

8) 8-21-97 PanA: Highly purified ECD-AP was presented to the library in solution form to try and isolate only high affinity binders (solution presentation of ECD-AP may minimize avidity effects which may occur when presenting target at high density on a matix) followed by incubation of the ECD-AP/phage solution with anti-AP Sepharose. Elution was by high and low pH. A total of four pans were performed.

9) 8-21-97 PanB: In contrast to the above pan which should limit "hits" to relatively high affinity binders, this pan was performed by adding highly purified ECD-AP to anti-AP plated at 10 ug/ml (high density) on 96-well polystyrene wells. This presentation may be advantageous for two reasons: a) it may allow identification of at least some binder, albeit low affinity, due to avidity effects and b) it may allow adjacent ECDs to be positioned closely enough on the matrix so as to assume the dimer conformation. It is possible, and is strongly suggested by the EPO and TPO work at Affymax, that presentation of the target in dimer form is necessary to obtain a reasonably high affinity binder. Elution was with high and low pH buffers. A total of four pans were performed.

Clone/sequence analysis

Enrichment: None of the pans showed more than a 2-4 fold enrichment over control target.

ELISA analysis of clones: Partially purified ECD-AP was plated on Nunc Maxisorb well at 10 ug/ml and parallel plates were set up plated with control protein (partially purified ECD-AP contaminants only)(Figure 14 or Figure 1, lanes 2 and 4). Some phage were also tested with AP only, as well as ECD-AP, on the wells. Incubation of the ECD-AP wells with Ab to the ECD demonstrated the presence of large amounts of active ECD-AP on the wells. The same Ab gave no signal when incubated on wells plated with Grb2-GST, streptavidin, or blocker alone. Phage known to bind to Grb2 and streptavidin bound well to their appropriate target and not to ECD-AP. (See "ELISA of phage clones" in the figure section). Therefore, both positive and negative controls for both target and phage appear to work well in the ELISA. Unfortunately, none of the phage isolated from the ECD-AP pans gave a positive signal by ELISA.

There is one possible problem with the ELISA in that some of the streptavidin (SA)-binding clones, which bear a consensus sequence known to bind SA (HPQF) [22] did not give a positive signal by ELISA. While it is possible that this consenus in those particular contexts do not bind with high enough affinity to be detected by ELISA, it has been demonstrated repeatedly that the HPQF sequence is a strong consensus for peptide binders to SA [22]. This brings our ELISA somewhat into question, despite the otherwise good performance of the other controls. Therefore, it is very possible that some of the consensus sequences described below are, in fact, ErbB2 ECD binding sequences, although perhaps not high affinity ones. While a positive ELISA signal would certainly make further analysis easier, the consensus sequences are likely to be valuable anyway in the construction of new biased libraries [5,6, 23] which are more likely to yield higher affinity ECD binders.

We will also attempt a competitive ELISA with phage clones using the two different monoclonal Abs to the ECD.

Sequence analysis of clones: See lists and alignments of amino acid sequences of phage clones isolated from pans in the figure section. Amino acids which occur at least twice in a vertical column are underlined. Only sequences with at least two amino acids identical to the consensus (albeit sometimes the consensus is degenerate with 2 or 3 amino acids) are entered into each alignment group. Additionally, the alignments contain many "similar" amino acids (most "degenerate" consensus amino acids are similar amino acids). Highlighting similar amino acids has been omitted for the sake of clarity. All sequences also have cysteines at each end in common, by design. These sequences were aligned by visual inspection. The sequences were also input into the Genetics Computing Group's "Pileup" program as well, both as individual pans and within pans. The new GCG Pileup algorithm gives more weight to certain amino acids, such as tryptophan, which may be appropriate for alignments of natural proteins, but is probably not optimal for our purpose. The "pileups" we obtained did not offer anything interesting we did not already detect by visual inspection. Visual inspection did yield interesting relationships not detected by Pileup. We will rerun the analyses on the old Pileup program and also the MEME program offered by the San Diego Supercomputing group.

As seen in the figures, while some pans did not identify any consensus sequences, some had strong or moderate consensus sequences. Some inter-pan consensus sequences were detected, sometimes from pans using two completely different forms of ErbB2 (whole native vs. ECD-AP). Such cross-pan consensus sequences are strongly suggestive that the sequences are binding to the only common element in the different presentation systems: ErbB2 ECD.

Searches: The "FINDPATTERNS" program from the GCG software package was used to search protein databases for similarity to our consensus sequences. No matches were found with either 0 or 1 mismatches allowed. The few that occurred with 2 mismatches (data not shown, but available on request) did not appear to be related to any protein we might expect, such as growth factors, receptors or AP substrates.

Live cell immuno-fluorescence assay (IFA): As discussed in the last report, we obtained a strong consensus (19 out of 20 clones were identical) by panning the library over live cells overexpressing ErbB2. We synthesized the peptide displayed by this clone and conjugated it to FITC. IFA using this FITC-peptide was negative. IFA of peptide-bearing phage was also negative. However, there are many variables in the assay that could account for a negative result: conjugation of FITC could render the peptide inactive; the monomeric binding of peptide labeled with a single FITC molecule may not be sufficient for detection of fluorescence; while, in our hands, Ab to the ECD appears to stay on the surface for at least 2 hours, bound peptides much smaller than Abs may be shed and/or endocytosed during the course of the assay, which may also be the case for phage particles much bigger than Abs. A more sensitive assay, using radioactive peptides, for example, may be necessary. We also plan to retry similar assays using fixed cells to avoid the above complications.

Biological effect assays: We have collaborators here at the UVM Department of Pharmacology who have systems established to routinely assess compounds for their effects on growth, viability, activation of ErbB2 and downstream targets such as MAP kinase, PI3-kinase, Raf-1, Jun kinase, and expression of *jun* and *fos*. Because of our negative ELISA results, we have

delayed these experiments. They will be initiated as soon as we obtain a peptide which binds with reasonable affinity.

Construction of improved random libraries and ErbB2 consensus mutagenesis libraries

Despite extensive screening with pure ErbB2 ECD, we have not obtained a binder from our present library which can be detected by ELISA. While there is possible fault with our ELISA, it is likely that new libraries, incorporating many of the improvements discussed in the introduction will greatly increase our chances of obtaining higher affinity binders. It is also important for us to try several different types of libraries. We designed five new libraries (designs are listed in the figure section) and have already begun their construction. The many degenerate oligos required for these libraries (listed in the figure section) are in the process of being synthesized by the DNA synthesis core facility here at UVM. We have already prepared large amounts of vector for Smith's gene III system and Affymax's peptides-on-plasmids system. The first library should be completed within the next 2 weeks, after which we will simultaneously pan the new library, which has become routine in our lab, while continuing with the construction of the other planned libraries.

We will also, depending on results of pans with the new random libraries, begin construction of mutagenesis libraries using the consensus sequences determined from past ErbB2 pans (see "Visual lineup of selected peptides isolated from ErbB2 pans" in the figure section).

Pans with other tumor targets

Muc-1 Pan: In a collaboration with O. J. Finn, who is a leading investigator of the promising tumor specific target, MUC-1, we screened our library with 100 mer MUC-1 provided by her laboratory. Sequences isolated from 3 separate pans are listed in the figure section. Unfortunately, no consensus was detected. One might imagine that the "knobby" [24] surface of the MUC-1 target might be better targeted by peptides from a library containing more concave surfaces rather than our library, which may be more "loopy". It is not hard to imagine "loops" having a hard time binding with much affinity to "knobs". Viewing conotoxin molecules in Rasmol (Protein Data Base) suggested that conotoxin peptides are more likely to present a surface which might "fit" over and bind to a knob. As mentioned in our original report, construction of such conotoxin libraries may be useful, especially for convex, rather than concave, targets. Alternatively, bigger and "better" random libraries, described in the Conclusions section of this report, may also yield MUC-1 binders. Such binders have the same promising potential as ErbB2 binders to specifically target breast tumor cells. We will certainly continue this exciting work once the new libraries are constructed.

CEA Pan/ Isolation of binders specific for polystyrene: CEA, "98% Pure" purchased from Vitro Diagnostics, Littleton, Co., was plated at 10 ug/ml in PBS on polystyrene Nunc Maxisorb wells on 96 well plates. While no consensus was isolated from the CEA wells, a strong consensus was detected from polystyrene wells coated with casein blocker only (see list of CEA pan sequences in the figure section). This was surprising, as no such consensus was detected from several other

pans which had ErbB2 ECD-AP or MUC-1 presented on polystyrene. These phage clones gave a strong ELISA signal over non-specific phage on wells blocked with either casein or 0.1% Tween (no casein present). While these binders may not be immediately relevant to breast cancer research per se, the finding has exciting potential application as a general research tool: these specific polystyrene-binding small peptides, assuming they don't displace bound target, may serve as a superior "blocker" for ELISA assays. In addition, if their affinity is high enough (and affinity may be increased, as for any peptide binder isolated through this technology), they could be linked to peptides or proteins and assist them in binding to plastic for ELISA assays, as some peptides and proteins do not bind well to polystyrene, or become denatured in the process.

The CEA pan will be repeated with new, improved libraries. In addition, the commercial CEA we used was analyzed by SDS-PAGE and contained several bands. A more pure preparation of CEA may optimize future screenings.

Grb2 binding peptide: Our work on the peptide we have identified which binds to the Grb2 SH2 domain has just been accepted by the Journal of Biological Chemistry, with Lyn Oligino from our lab as first co-author. Additional data not reported last year include the finding that phosphorylation of the Tyr residue of G1 increased binding to an even higher level than the SHC phosphopeptide, further evidence that G1 binds directly in the phosphopeptide binding pocket for the Grb2 SH2 domain. An exciting development in our progress on this peptide is that the directly cyclized version of G1 inhibits the interaction of Grb2 with its major intracellular targets in cell lysates. Such an inhibitor to the ErbB2 and other signal transduction pathways has considerable promise as an anti-cancer agent. It is especially promising that the peptide works in the context of cell lysate, complete with its enzymes and reducing agents. This data suggests that G1 may work inside the cell itself, once we succeed in getting it there.

We plan to construct a mutagenesis library in the near future using the G1 library as a consensus. Again, when the group from Affymax used low affinity consensus sequences as a basis for mutagenesis libraries, they obtained very high affinity binders. We are hoping we will be as successful with G1.

Isolation of aptamers to ErbB2

The aptamer aspect of our project had been put on hold until sufficient quantities of highly purified ErbB2 ECD were available. Now that adequate pure target is available, the project has been revitalized. Detailed protocols have been designed, and work should begin in the next few weeks. In addition, we have acquired the services of a new technician expert in PCR to assist in this project.

Conclusions

Task 1. Construct small ligand libraries. It is clear from our results this year, and especially upon review of the recent exciting successes of Affymax discussed in the introduction, that new libraries need be constructed. Five new libraries have been designed (FIG) and construction is

underway. The libraries will be of a much greater size than our first library (10^{10} to 10^{11}) and will contain disulfide loops of various size (8-12 amino acids). The disulfide loop will be flanked on both sides by 4 random amino acids. We will use two different gene III (minor coat protein) phage display systems, a gene VIII (major coat protein) system, a Lac repressor system (peptides-on-plasmids), and the "headpiece dimer" system (monomeric form of the peptides-on-plasmids system). These libraries will allow us to screen in both low affinity modes and high affinity modes, the importance of which is discussed abovel

In addition, mutagenesis libraries will be constructed using the consensus sequences determined from our numerous ErbB2 pans.

Task 2. Develop methods to affinity isolate ligands which bind to ErbB2, a cell surface protein associated with breast cancer.

Affinity isolation of peptide ligands to ErbB2 from our library has become routine in our lab. We have become expert in several forms of presentation of target including binding the target covalently and noncovalently to matrices, densely arrayed and in solution, linked to a matrix via an Ab or not. We routinely use several different matrices including Sepharose, polystyrene, paramagnetic particles or beads, and nitrocellulose. While it is likely that any of these methods will work once the libraries contain the binders we seek, it is to our advantage to have all these techniques at our disposal, as different presentation systems sometimes work better for different targets, for reasons not yet understood. We will also further explore the use of ErbB2-overexpressing live and fixed cells for affinity purifications.

Presentation and screening can now be performed with much more confidence and facility now that we have available ample amounts of highly purified ErbB2 ECD.

We also this year determined that treatment of phage with air oxidation or DMSO did not affect viability of phage. Considering the importance of the disulfide loops, we will further experiment with this technique: such treatment might facilitate the formation of disulfide loops on the phage-displayed peptides and optimize our chance of obtaining a hit.

Recent improvements in presentation and elution schemes, as discussed in the introduction, such as presentation via Ab, preelution with a known ligand, and elution by thrombin cleavage of the receptor will also be incorporated into our screenings, if necessary.

We have also developed an assay useful for investigating the process of ErbB2 dimerization, thought to be essential for signal transduction. In a collaboration with Marc Lippman at Georgetown, we will test the ability of small peptides derived from the ECD of ErbB2 to inhibit dimerization. Such peptides have the potential for therapeutic utility.

Task 3. Isolate and characterize peptide-phage and ssDNA molecules which bind to ErbB2.

We have performed extensive panning experiments with purified ErbB2 this year. Although we have still not identified a clone with binds to ErbB2 using our present ELISA assay, we have obtained consensus sequences which may prove very useful in obtaining higher affinity binders through the construction of mutagenesis libraries using the consensus sequences as core sequences. The panning procedures are now routine in our lab, and once the new libraries, already under construction, are complete, we should be able to rapidly "plug" them into our panning procedures. We believe the new libraries will have a much greater chance of yielding

binders, as similar libraries at Affymax were successful in yielding binders to receptors very similar to ErbB2.

Task 4. Synthesize and characterize small ligands to ErbB2.

We synthesized a peptide identified from live ErbB2-overexpressing cell pans and conjugated it to FITC. Unfortunately, an IFA using this peptide was negative. Possible reasons for this failure, even if the peptide does bind to ErbB2, include possible shedding or cell internalization of the bound peptide, lack of a stong enough fluorescent signal from peptide monolabeled with FITC, or destruction of peptide binding activity after conjugation with FITC. The first of these possible failures may be ameliorated by the use of fixed cells. Radiolabeled peptides, such as peptides labelled with ^{35}S cysteines, may prove more useful for subsequent characterization.

As discussed in last year's report, we have synthesized Grb2 binding peptides and analogues, which has been extensively characterized in our recently acccepted publication [7]. While the focus of this project is not Grb2, but ErbB2, the Grb2 work has allowed us to become expert at the techniques necessary for characterization of peptide ligands in general. We have established many valuable collaborations at NIH and elsewhere, that will hopefully allow us to rapidly complete Task 4 once higher affinity ligands are identified.

Synthesis, cyclization, HPLC analysis, and mass spectroscopy analysis has now become fairly routine in our lab, and again, will allow rapid completion of Task 4 once high affinity ErbB2 ligands are identified from the new libraries.

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Figure 1. Silver-stained SDS-PAGE of partially purified ECD-AP showing 3-4 major contaminants.
Lanes 1 and 3: Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP sup. The top band at 150kd is ECD-AP, as verified by Western blot experiments.
Lanes 2 and 4: Protein eluted from anti-AP Sepharose incubated with 3T3 control sup.

Figure 2. Silver-stained SDS-PAGE of fractions #1-30 from a preparative SDS-PAGE electophoresis run. ECD-AP appears in the last 2 fractions.

Figure 3. Silver-stained SDS-PAGE of fractions #1-30 from a preparative native electrophoresis run.
Lanes 9-30: ECD-AP

Figure 4. Coomassie-stained SDS-PAGE of fractions #1-30 from an ion exchange FPLC run.
Lane 8: ECD-AP
Lane 9: Top major contaminant (some ECD-AP)

Figure 5. Coomassie-stained SDS-PAGE of lectin-Sepharose affinity experiments with ECD-AP.
Lanes 1-2: ConA ECD-AP
Lanes 3-4: ConA 3T3 control
Lanes 6-7: Lentil ECD-AP
Lanes 8-9: Lentil 3T3 control
Lanes 13-14: Wheat germ ECD-AP
Lanes 15-16: Wheat germ 3T3 control
Odd lanes were eluted with a low sugar concentration; even lanes were eluted with a high sugar concentration.

Figure 6: Coomassie-stained SDS-PAGE of ECD-AP partially purified (pp) by anti-Ap Sepharose affinity purification followed by lectin-Sepharose purification.
Lane 2: Protein remaining in pp ECD-AP post incubation with ConA- Sepharose
Lane 4: Protein remaining in pp ECD-AP post incubation with lentil-Sepharose
Lane 6: Protein remaining in pp ECD-AP post incubation with wheat germ-Sepharose
Lane 8: Protein remaining in pp ECD-AP post incubation with Protein A-Sepharose
Lane 11-12: Protein bound to ConA
Lane 14-15: Protein bound to lentil
Lane 17-18: Protein bound to wheat germ
Odd lanes were eluted with a low sugar concentration; even lanes were eluted with a high sugar concentration.
Lane 20: Protein bound to Protein A

Figure 7: Silver stained *native* PAGE shows ECD-AP dimer.
Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP sup.

Figure 8: Coomassie stained *native* PAGE shows ECD-AP dimer.
Left lane: Protein eluted from anti-AP Sepharose incubated with 3T3 control sup
Right lane: Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP sup

Figure 9: Western blot of *native* PAGE shows ECD-AP dimer
Left lane: Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP sup
Right lane: (No signal) Protein eluted from anti-AP Sepharose incubated with 3T3 control sup

Figure 10: Coomassie stained *native* PAGE shows multimeric ECD-AP.

Figure 11: Coomassie stained *native* PAGE shows pure AP running as one band only.
Left lane: Pure AP
Right lane: Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP sup

Figure 12: Coomassie stained *native* PAGE shows no effect of excess AP on ECD-AP dimers.
Lane 1: ECD-AP with no AP present
Lane 2: 5 ug AP
Lane 3: ECD-AP incubated with 0.5 ug AP
Lane 4: ECD-AP incubated with 1.0 ug AP
Lane 5: ECD-AP incubated with 5.0 ug AP
Lane 6: ECD-AP incubated with 10 ug AP

Figure 13: Coomassie stained SDS-PAGE of ECD-AP electroeluted from a native gel.
Lane 1: Partially purified ECD-AP from anti-AP Sepharose
Lane 2: Top (dimer) band of ECD-AP eluted from a native gel
Lane 3: Bottom (monomer) band of ECD-AP eluted from a native gel

Figure 14: Serum is the source of the major contaminants.
Lanes 1 and 2: Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP sup
Lane 3: (No signal) Protein eluted from anti-AP Sepharose incubated with DMEM without serum
Lane 4: Protein eluted from anti-AP Sepharose incubated with DMEM with serum

Figure 15: Pure ECD-AP from SFM.
Protein eluted from anti-AP Sepharose incubated with 3T3/ECD-AP SFM sup

Figure 16: Increased ECD-AP production in roller bottles
Lane 1: 1/20 third overlay harvest
Lane 2: 1/20 fifth overlay harvest
Lane 3: Initial harvest

Figure 17: Pure ECD-AP filtered through YM100 membranes
Lane 1: Concentrated harvest
Lane 2: Filtrate

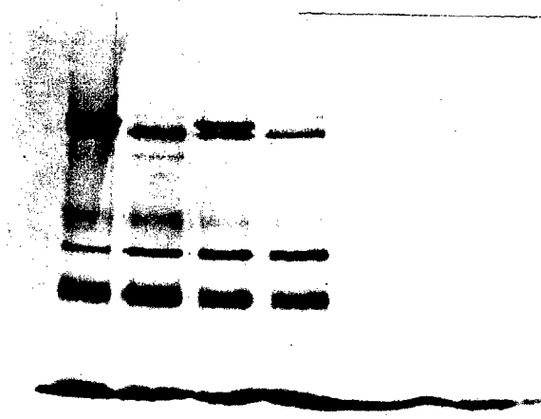
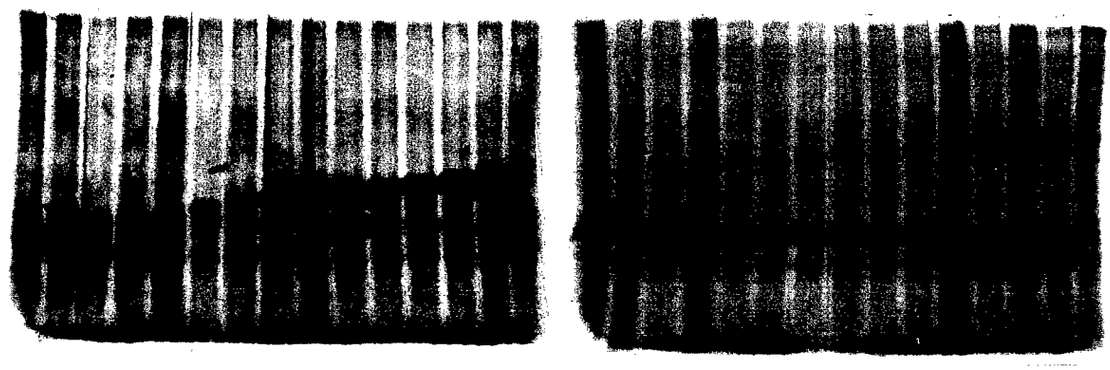


Figure 1



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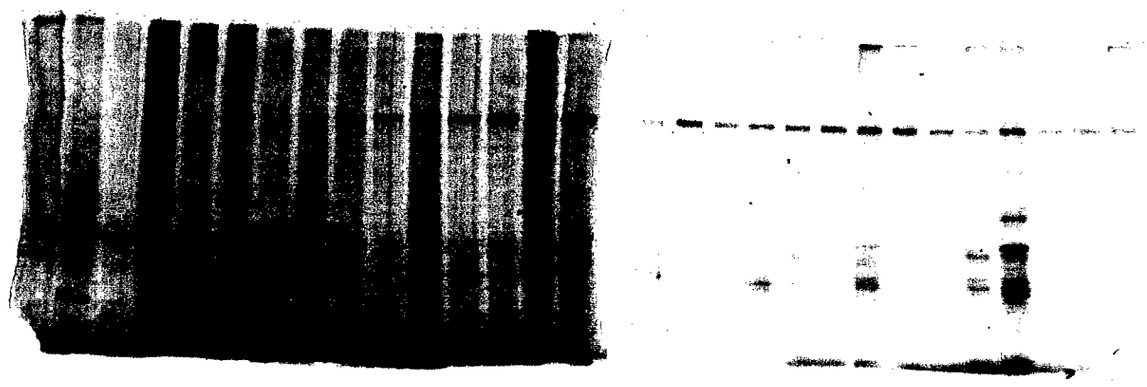
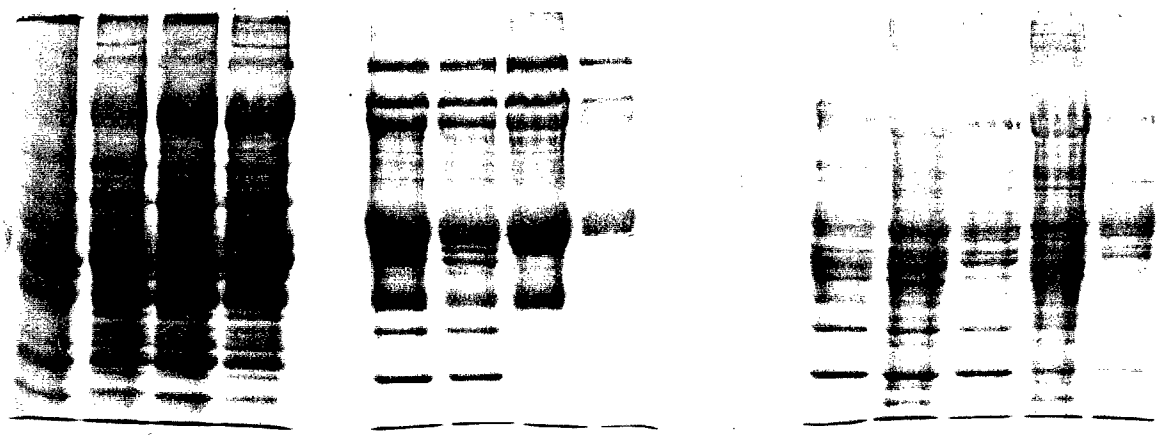


Figure 3

Figure 4



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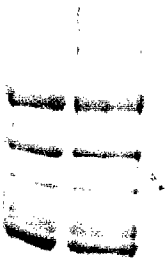

Figure 6

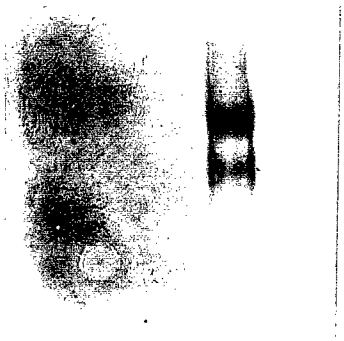

Figure 7

Figure 8

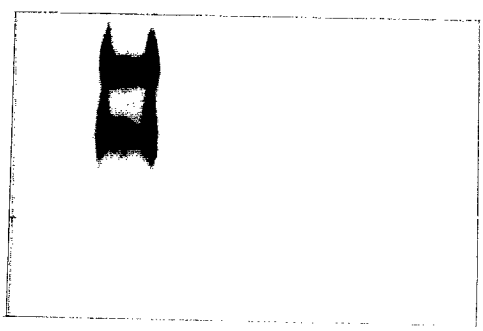

Figure 9

Figure 10

Figure 11

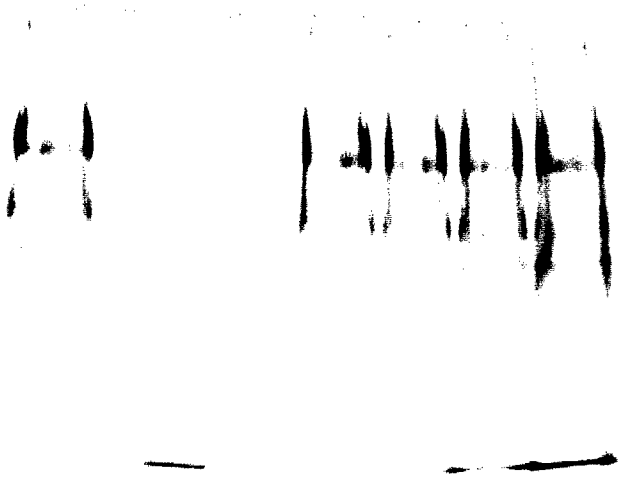

Figure 12

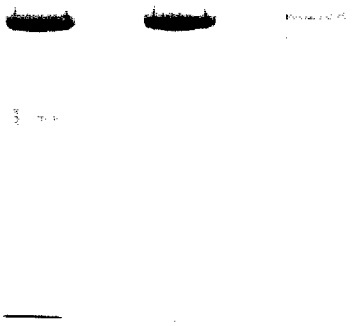

Figure 13

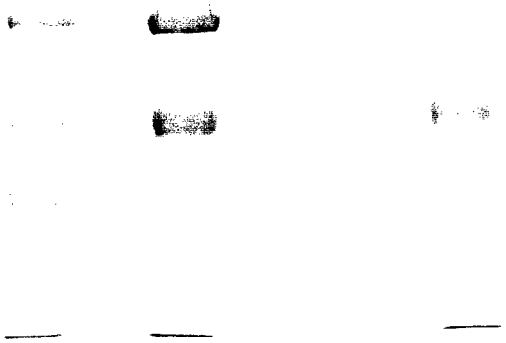

Figure 14

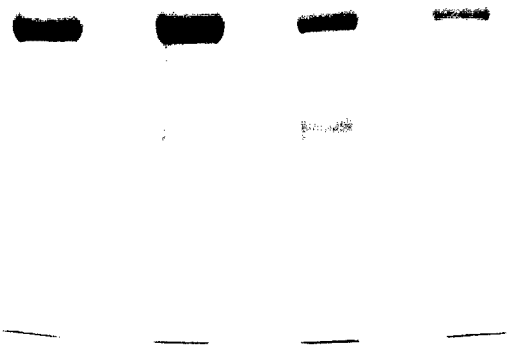

Figure 15

Figure 16

Figure 17

Visual lineup of selected peptides isolated from ErbB2 pans:

QLLKLSTYG		3-16-97
GQLLSWMDM		12-5-95
LQSNLLRFM		7-22-94
NLLAIMARS		12-5-95
NLLAIMARS		7-22-94
NLLEMMGFV	(3)*	7-22-94
SALDYMTRS	(9)	3-22-95
SALDYMTRS		12-5-95
SLDRYDHFG		3-22-95
MAAIQTDRG	(2)	3-22-95
IVNRKSILD		3-16-97
DFILDLDLS		7-21-94
LVSKGRSLD		5-12-97
LNRLLSEGK		9-8-97
IQMGRSPAI		4-1-97

Distribution Limited

Note: Q,S are similar to N; A is similar to L; D is similar to E.

VHWWLEING	(2)	8-21-97
VHLELTPSW		4-16-97
VHVELTHSW		4-16-97
AWNSVMTFG	(2)	5-12-97
GMGLSTFYN		5-12-97
VEYFELGLS		5-12-97
FELMRGQIA		5-12-97
WLDELMSHL		12-5-95
GQMRAMLGL		12-5-95
KVMAMTLGI		12-5-95
YASAFFPWS		5-26-97
YWGEFFSRV	(16)	2-5-97
M-QSQWG--F		4-1-97
WVKGEFPRD		8-21-97

*Numbers in parentheses after the sequence indicate the number of clones isolated if more than one.

Note: Pans dated 97 and pre-97 have only ErbB2 ECD in common in the proteins of the target presentation system. Some have a common matrix (Sepharose) although other cross-pan similarities are from targets presented on completely different matrices.

Peptides displayed by clones isolated from the ECD-AP/αAP-Sepharose pan 2-5-97

	Number of clones
Y W G E F F S R V	16

Peptides displayed by clones isolated from the Nitrocellulose-ECD-AP pan 3-16-97

```
e      A S P I S V L E L
e              Q L L K L S T Y G
e      G R G H Y L L P V
e    I V N R K S I L D
c          K E D L T D E S D
e                    P D R S Q V G P M

c          V G F G R A L V R
e    N W T T S F G W G
c              F G T G E Q F G A
e    F S W L S W H D G
c      S Y S V W Y D S W
c      E W V S T Y P Y S

c    N R R W P S G Q N
c        V M P S G G A I L

e    V Q S A W M T H D
c    V F H P Q N L S G
```

e=clones eluted from ECD from the last pan.
c=clones eluted from control target from the last pan. They are included since, if any enrichment did take place during pans 1 and 2, ECD binders would also be expected to be eluted from any pan 3 target.

Peptides displayed by clones isolated from the Polystyrene-ECD-AP pan 4-1-97

```
          I Q M G R S P A I
          G A M S Q S Q N Q
          V K M E Q A Q W W
          E G R L Q G L W G
            S P W K W L W G W
              A I R P V W G G I
                        W G I P F A R A H
                  V D V A T G T G F

Consensus:    M - Q S Q W G - - F
              ~ ~ R ~ ~ ~ ~
              ~ ~ ~ ~ L ~ ~
```

Peptides displayed by clones isolated from the Nitrocellulose-ECD-AP pan 4-16-97

```
d     V H L E L T P S W
db    V H V E L T H S W

db    A G G S S P P L R
m           S S I F S S G I L

d     E W S R T T T W L
d     S K T M P A V S T
d     G L A R G M P F S
d     L P S G Q L I P M
db    W R V C N S D R C
m     T H A A S R N V S
m     M S P G T L G G P
m     N L E A W T S V K
m     E R R I G T F L R
mb    E P K R S G G F T
mb    K V R T H Y N W Q
mb    G E P L L W L Y L
```

d=dimer pan
m=monomer pan
b=clones eluted from the blank NC control of the last pan; see footnotes to 3-16-97 pan alignment.

Peptides displayed by clones isolated from the Sepharose-ECD-AP pan 5-12-97

A W N S V M T F G
A W N S V M T F G
E M L I P R T T F
G M G L S T F Y N
V E Y F E L G L S
F E L M R G Q I A
*W L D E L M S H L
C K Q C V A S F L
R G V G P A S G A

L V S K G R S L D
S L I T V D T P R

*Note: this peptide is from a past whole ErbB2 pan without AP.

Peptides displayed by clones isolated from the Dynabead-ECD-AP pan with Dynabead-AP control and preclear 5-26-97

	Number of clones
M V T L E S W P R	3 (1e,2a)
K K V W C V S G V	3 (a)
E D P W G G L V T	2 (1e,1a)
G W E T I Y M N L	2 (e)
Y A S A F F P W S	1 (a)
F N V T G W R M V	1 (e)
D G Y W R F W A G	1 (e)

e=clones eluted from ECD-Dynabeads after the last pan.
a=clones eluted from AP-Dynabeads after the last pan. See footnotes to 3-16-97 lineup.

Peptides displayed by clones isolated from the Polystyrene-ECD-AP pan 7-21-97

A P T H L R L A T
M E R T P H A V A
L G G R R W M A M
M V R A G W K K T
V A Y Q S Y V P S
R L P S Q V W H K
G W T R S P E V F
P V T D V D I S V
P P S W G A V W E
M F T S F V G R S
A T L R E H P W A
E V R G L V W E P
F G P A Y I A A T
G K D V A M L T W
G S M I V Y T R Y
R D K S P Q T A Y

Peptides displayed by clones isolated from the ECD-AP pan A 8-21-97

V H W W L E I N G
V H W W L E I N G

A F W V E A E L V
W V K G E F P R D

N G I T L G I T W
G H V S D L T S H
Q H I Q A D R F Q
V V R P S Y H Y L
S R D L Q V V L N
S Q F H S A Y D R
Y G M H F R G G M
M Y T S S Q F T W
G S S Y L F G V D
H H S G T Q P V S
V E N G Y G I A T
G D M W K V T A S

Peptides displayed by clones isolated from the Polystyrene-αAP-ECD-AP pan B 8-21-97

S G R S G E A T V
M V T A R S G V F
L T S L P E R R S
I F S G D W M V P
N F R E G V S G A
D L I Q Y R L G P
A G I R T L A G N

S L F H N Q A M G
D V S I F P R D V
G K W S P Y M R R
L A S M P L Q I E
M F P G V R D D T
I W G R G L A P M
D E Y V L R R H L
L N R L L S E G K
L P L K G P T R L

Peptides displayed by clones isolated from the MUC-1 pan 6-19-97

m					I	Q	W	N	M	S	F	P	S
p		K	S	T	P	K	V	W	N	M			
m		L	L	W	D	V	S	N	L	D			
m		F	N	T	V	L	I	G	W	G			
m		Y	G	V	V	W	S	A	V	K			
m		M	S	T	V	K	G	F	S	L			
m		A	P	S	S	R	W	L	G	P			
m		D	G	L	M	P	R	W	L	Q			
p		W	T	G	T	R	Y	G	Y	L			
p		I	F	F	D	F	R	H	V	K			
p		W	M	L	N	R	M	Y	L	S			
p		L	L	S	T	A	M	R	E	V			
p		V	L	Q	G	L	L	L	P	S			
p		V	D	M	G	I	S	P	V	Q			
p		T	G	L	N	V	V	H	I	H			

m=clones eluted from MUC-1after the last pan.
p=clones eluted from the polystyrene only control after the last pan. See footnotes to 3-16-97 lineup.

Peptides displayed by clones isolated from the MUC-1 pan 6-24-97

I	L	G	M	L	W	Q	S	K
F	R	W	E	L	G	R	V	L
E	R	W	N	T	T	L	D	R
V	L	A	S	L	I	D	G	S
D	S	A	F	M	G	T	N	L
S	H	R	G	K	W	S	Y	N
F	H	L	N	K	N	P	T	Y

Peptides displayed by clones isolated from the MUC-1 pan with αMUC-1 Ab elution followed by pH elution 7-8-97

ab	G	P	L	V	D	E	A	H	R
ab	I	P	Y	R	L	G	F	L	N
ab	G	L	L	E	V	S	G	R	R
ab	V	G	V	Q	C	N	R	S	M
ab	Y	P	P	N	N	P	L	L	F
ab	K	V	L	S	I	K	G	G	V
ab	Y	S	H	S	A	D	G	A	C
ab	V	T	S	Y	L	H	S	S	I
ab	E	V	N	P	N	V	G	A	R
ab	Q	R	L	S	F	P	S	A	M
ab	M	I	D	P	Y	S	Y	S	A
ab	T	G	F	R	L	T	R	H	L
ab	L	R	V	M	M	E	P	N	V
ab	P	G	R	N	S	P	V	Y	R
ab	M	G	S	C	S	S	K	T	L
ab	L	N	T	G	M	S	S	R	N
ph	G	Q	P	L	V	L	E	L	G
ph	R	E	A	F	Y	G	I	R	E
ph	R	L	T	S	W	D	E	L	M
ph	E	R	S	M	L	S	S	L	I
ph	S	V	T	S	R	G	W	L	L
ph	T	S	R	V	S	L	A	T	G
ph	I	W	M	G	L	L	G	V	P
ph	W	G	E	V	G	E	K	G	N

ab= clones isolated after anti-MUC1 Ab elution from the last pan.
ph= clones isolated after high and low pH elution from the last pan.

Peptides displayed by clones isolated from the CEA pan

c	V	L	K	V	G	W	L	S	A
c	Y	P	L	G	S	P	R	F	K
c	W	K	G	E	A	H	M	P	Y
ps	R	H	W	N	Y	F	F	G	W
ps	R	H	W	N	Y	F	F	G	W
ps	R	Y	K	H	F	N	F	P	W
ps	R	L	M	G	L	N	R	G	W

ELISA with phage clones

	antigen (1 μg/well)				
	ECDAP	AP	blank	Streptavidin	GRB2
phage					
clone from ECD-AP pan 2-5-97	0.147	0.069	0.062	ND	ND
clone from ECD-AP pan 2-5-97 1:10	0.169	0.085	0.074	ND	ND
clone from Streptavidin pan	0.134	0.101	0.061	0.062	ND
clone from Streptavidin pan 1:10	0.146	0.067	0.060	**0.737**	0.125
clone from GRB2 pan	0.142	ND	0.109	0.095	**2.125**
clone from GRB2 1:10	0.157	ND	0.103	0.078	**1.880**
Anti-ErbB2 ECD-antibody	**2.258**	ND	0.067		

NOTE: 1:10 dilutions of phage were in PBS.
Undiluted phage from Streptavidin pan does not react to due to competition from biotin in media.
ND = not done

New Library Designs 8/97

Lac repressor (peptides-on-plasmids) library:

X_4 C X_{8-12} C X_4

Headpiece dimer library:

X_4 C X_{8-12} C X_4

Gene VIII library:

X_4 C X_{8-12} C X_4

Gene III, fuse 5 library (Smith):

X_4 C X_{8-12} C X_4

Gene III, fAFF1 library (Dower):

X_4 C X_{8-12} C X_4

X= random amino acid residues.

Oligos required by our lab- Page 1 of 2

Lac library: (All are 5'-phosphorylated)

ON-829
ACC ACC TCC GG

ON-830
TTA CTT AGT TA

Lac-library oligo 8
GA GGT GGT $\{NNK\}_4$ TGC $\{NNK\}_8$ TGC $\{NNK\}_4$ TAA CTA AGT AAA GC

Lac-library oligo 9
GA GGT GGT $\{NNK\}_4$ TGC $\{NNK\}_9$ TGC $\{NNK\}_4$ TAA CTA AGT AAA GC

Lac-library oligo 10
GA GGT GGT $\{NNK\}_4$ TGC $\{NNK\}_{10}$ TGC $\{NNK\}_4$ TAA CTA AGT AAA GC

Lac-library oligo 11
GA GGT GGT $\{NNK\}_4$ TGC $\{NNK\}_{11}$ TGC $\{NNK\}_4$ TAA CTA AGT AAA GC

Lac-library oligo 12
GA GGT GGT $\{NNK\}_4$ TGC $\{NNK\}_{12}$ TGC $\{NNK\}_4$ TAA CTA AGT AAA GC

Gene VIII library: (*not* phosphorylated)

ON-1592
T AGG GCC CAC CTT GCT GGG ATC GTC GGA GCC ACC TCC CCC ACT TCC CCC ACC GCC GCT ACC CCC GCC TCC

Gene VIII library oligo 8
GGC CCA GTG CTC ACG CA $\{NNK\}_4$ TGC $\{NNK\}_8$ TGC $\{NNK\}_4$ GGA GGC GGG GGT AGC

Gene VIII library oligo 9
GGC CCA GTG CTC ACG CA $\{NNK\}_4$ TGC $\{NNK\}_9$ TGC $\{NNK\}_4$ GGA GGC GGG GGT AGC

Gene VIII library oligo 10
GGC CCA GTG CTC ACG CA $\{NNK\}_4$ TGC $\{NNK\}_{10}$ TGC $\{NNK\}_4$ GGA GGC GGG GGT AGC

Gene VIII library oligo 11
GGC CCA GTG CTC ACG CA $\{NNK\}_4$ TGC $\{NNK\}_{11}$ TGC $\{NNK\}_4$ GGA GGC GGG GGT AGC

Gene VIII library oligo 12
GGC CCA GTG CTC ACG CA $\{NNK\}_4$ TGC $\{NNK\}_{12}$ TGC $\{NNK\}_4$ GGA GGC GGG GGT AGC

Oligos required by our lab- Page 2 of 2

Gene III, fuse 5 library: (All are 5'-phosphorylated)

Gs5' oligo
TCC GCC AGC CCC GT

Gs3' oligo
C GGC CCC GCC TCC

Gs library oligo 8
GG GCT GGC GGA $\{NNK\}_4$ TGC $\{NNK\}_8$ TGC $\{NNK\}_4$ GGA GGC GGG GCC GCT G

Gs library oligo 9
GG GCT GGC GGA $\{NNK\}_4$ TGC $\{NNK\}_9$ TGC $\{NNK\}_4$ GGA GGC GGG GCC GCT G

Gs library oligo 10
GG GCT GGC GGA $\{NNK\}_4$ TGC $\{NNK\}_{10}$ TGC $\{NNK\}_4$ GGA GGC GGG GCC GCT G

Gs library oligo 11
GG GCT GGC GGA $\{NNK\}_4$ TGC $\{NNK\}_{11}$ TGC $\{NNK\}_4$ GGA GGC GGG GCC GCT G

Gs library oligo 12
GG GCT GGC GGA $\{NNK\}_4$ TGC $\{NNK\}_{12}$ TGC $\{NNK\}_4$ GGA GGC GGG GCC GCT G

Gene III, fAFF1 library: (All are 5'-phosphorylated)

Dower ON-28
GGA GTG AGA GTA GA

Dower ON-29
CTT TCA ACA GT

fAFF1 library oligo 8
C TCT CAC TCC $\{NNK\}_4$ TGC $\{NNK\}_{12}$ TGC $\{NNK\}_4$ GGC GGC ACT GTT GAA AGT TGT

fAFF1 library oligo 9
C TCT CAC TCC $\{NNK\}_4$ TGC $\{NNK\}_9$ TGC $\{NNK\}_4$ GGC GGC ACT GTT GAA AGT TGT

fAFF1 library oligo 10
C TCT CAC TCC $\{NNK\}_4$ TGC $\{NNK\}_{10}$ TGC $\{NNK\}_4$ GGC GGC ACT GTT GAA AGT TGT

fAFF1 library oligo 11
C TCT CAC TCC $\{NNK\}_4$ TGC $\{NNK\}_{11}$ TGC $\{NNK\}_4$ GGC GGC ACT GTT GAA AGT TGT

fAFF1 library oligo 12
C TCT CAC TCC $\{NNK\}_4$ TGC $\{NNK\}_{12}$ TGC $\{NNK\}_4$ GGC GGC ACT GTT GAA AGT TGT

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 10 Jul 00

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2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at Virginia.Miller@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management